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Annual Report

PCRP Predoctoral Traineeship Award

W81XWH-06-1-0015

Stathmin: A “Relay Protein” in the Development of Prostate Cancer and a Potential Target for Anti-cancer Therapy

P.I. Ritwik Ghosh, M.S.

Introduction

The **long term goal** of this work is to determine whether stathmin can be targeted as an effective therapy in the clinic against prostate cancer. The **central hypothesis** of this proposal is that *overexpression of stathmin promotes prostate cancer development and blocking stathmin expression sensitizes prostate cancer cells to anticancer therapies such as Taxotere and Erbitux*. The **purpose** of this work is to i) correlate stathmin overexpression with progression of prostate cancer, ii) determine the signaling pathways activated through selective phosphorylation of stathmin and whether inactivation of these pathways promotes sensitization to treatment with Taxotere or Erbitux and iii) examine the effects of stathmin expression on tumor development and the outcomes of Taxotere, Erbitux on blocking tumorigenesis in tissue recombination and transgenic mouse models. The **rationale** is to develop combinatorial treatment strategies for better clinical management of prostate cancer patients. Targeting stathmin in prostate cancer can potentially sensitize patients to treatment with Taxotere or Erbitux. Since the agents selected have already been used in the clinic, successful outcomes in the animal models can result in rapid clinical trials.

Statement of Work

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Stathmin: A “Relay Protein” in the Development of Prostate Cancer and a Potential Target for Anti-cancer Therapy

P.I. Ritwik Ghosh, M.S.

Task 1

Investigate how the level of stathmin expression regulates tumorigenesis in prostate cancer cells

- a) Study stathmin expression by immunohistochemistry in tissue arrays containing low grade and high grade human prostate tumor samples from 111 patients to analyze and semi-quantify the levels of stathmin expression as they increase with cancer grade. The levels of stathmin expression to be compared to that in benign prostatic hyperplasia (BPH).

Status: Stathmin expression has been studied in tissue arrays arranged according to Gleason Pattern. The initial immunohistochemistry on a tissue array containing 111 patient samples exhibited background staining and hence was not fit for any statistical analyses. New tissue arrays were procured from The Prostate Cancer Center at Vancouver General Hospital, University of British Columbia, British Columbia, Canada. These tissue arrays contained 54 cores from 34 BPH and prostate cancer patients. The cores were arranged according to Gleason pattern. Stathmin expression in these cores have been studied and analyzed.

- b) Study consequence of stathmin overexpression or loss of expression on proliferation, migration and invasion using NeoTag1 and NeoTag2 cells.

Status: Stathmin expression has been successfully knocked down in NeoTag1 and NeoTag2 cells using SMARTpool siRNA from Dharmacon. In an article published in December 2005, Mistry et al. showed that knocking down stathmin expression in androgen-independent LNCaP cells caused a cell-cycle arrest, induced apoptosis and suppressed clonogenic potential of the cells [1]. Therefore, we knocked down stathmin and analyzed the NeoTag1 and NeoTag2 cells for changes other than those published for LNCaP cells. In addition, we knocked down stathmin in LNCaP, PC-3 and DU145 cells. Surprisingly, in the DU145 cells, knocking down of stathmin induced an EMT (Epithelial to Mesenchymal Transition)-like phenotype. Of all the prostate cancer cell

lines, only DU145 undergoes TGF β induced EMT like phenotype. Knocking down stathmin sensitized the cells to TGF β -induced cellular morphology changes resulting rapidly in an EMT-like phenotype. We found that stathmin may be modulating epithelial to mesenchymal transition of DU145 cells. Since this is a key event in invasion and subsequent metastasis in the tumor microenvironment, we postulate that stathmin may be involved in tumor metastasis. We are presently studying the effects of stathmin in regulating various molecular pathways in this EMT phenotype.

Task 2

Determine which signaling pathway is activated through selective phosphorylation of stathmin and whether inactivation of this pathway promotes sensitization to treatment with Taxotere or Erbitux.

Status: Phospho-antibodies against specific serine residues of stathmin were used to study phosphorylation pattern in NeoTag1 and NeoTag2 cells. We also studied phosphorylation in LNCaP, PC-3 and DU145 cells. All small molecule kinase inhibitors initially proposed failed to knock down stathmin phosphorylation in all the cell lines tested. Hence, as an alternative we designed primers to generate phosphorylation site mutants to knock down stathmin phosphorylation in these cells lines. Generation of plasmids containing phosphorylation site mutant stathmin sequence and subsequent expression of these plasmids in the cells is pending.

Task 3

Evaluate the effects of stathmin expression on tumor development and the outcomes of Taxotere, Erbitux treatment on blocking tumorigenesis and metastasis in tissue recombination and transgenic mouse models.

Status: Not yet Started.

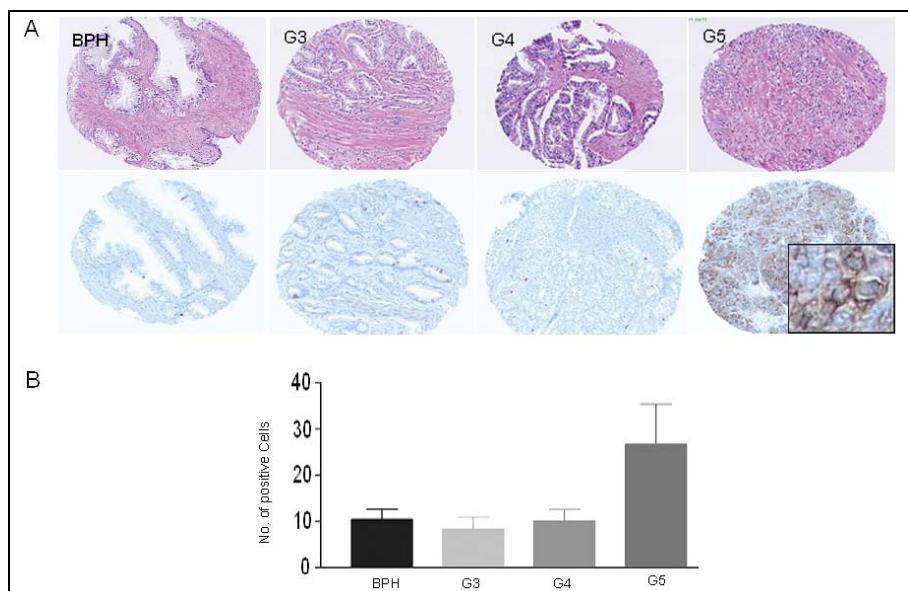
Summary of the Project

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Stathmin: A “Relay Protein” in the Development of Prostate Cancer and a Potential Target for Anti-cancer Therapy

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The main tasks for the first year of this proposal have been, 1) Correlate stathmin expression with progression of human prostate cancer and 2) Investigate the consequence of stathmin overexpression or loss of expression on proliferation, migration and invasion in prostate cancer cells. In a tissue array containing human prostate cancer specimens we found stathmin increases



UNPUBLISHED DATA

Figure 1. Immunohistochemical analyses of stathmin showing increased stathmin expression correlates with increasing Gleason pattern.

A. H&E staining and staining with anti-stathmin antibody. **B.** Quantitative analyses of stathmin expression with increasing Gleason pattern.

with Gleason pattern in a statistically significant manner (Fig. 1). This tissue array has been organized according to Gleason Pattern and has 54 cores from 34 patients. The array has specimens from BPH and Gleason patterns 3, 4 and 5 patients. Stathmin expression was higher in Gleason pattern 5 compared to Gleason pattern 4 ($p < 0.05$), Gleason pattern 3 ($p < 0.01$) and BPH ($p < 0.05$) (Fig.1). We also performed immunohistochemical

analyses for stathmin expression on a tissue array array containing cores from 111 prostate cancer patient samples. However, the staining pattern had too much background to perform proper quantification and statistical analyses.

Stathmin expression was also analyzed in tissue recombinants with NeoTag1 and NeoTag2

cells. In the tissue recombination model, NeoTag1 cells form mostly PIN and limited adenocarcinoma. NeoTag2 tissue recombinants form mostly adenocarcinoma and some PIN. In the NeoTag1 tissue recombinants, stathmin expression was higher in the PIN lesions compared to

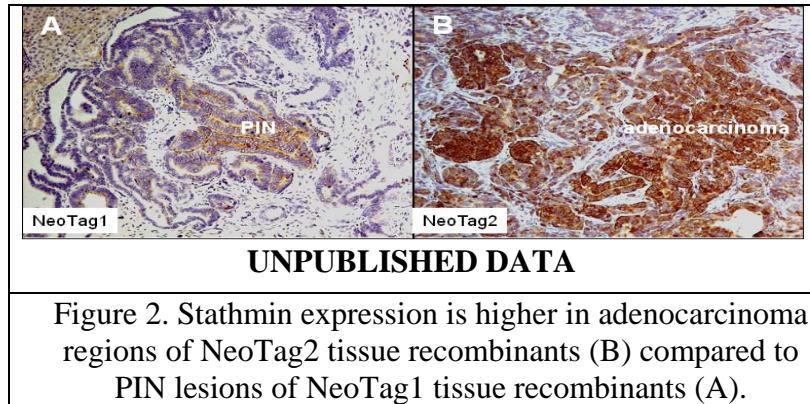
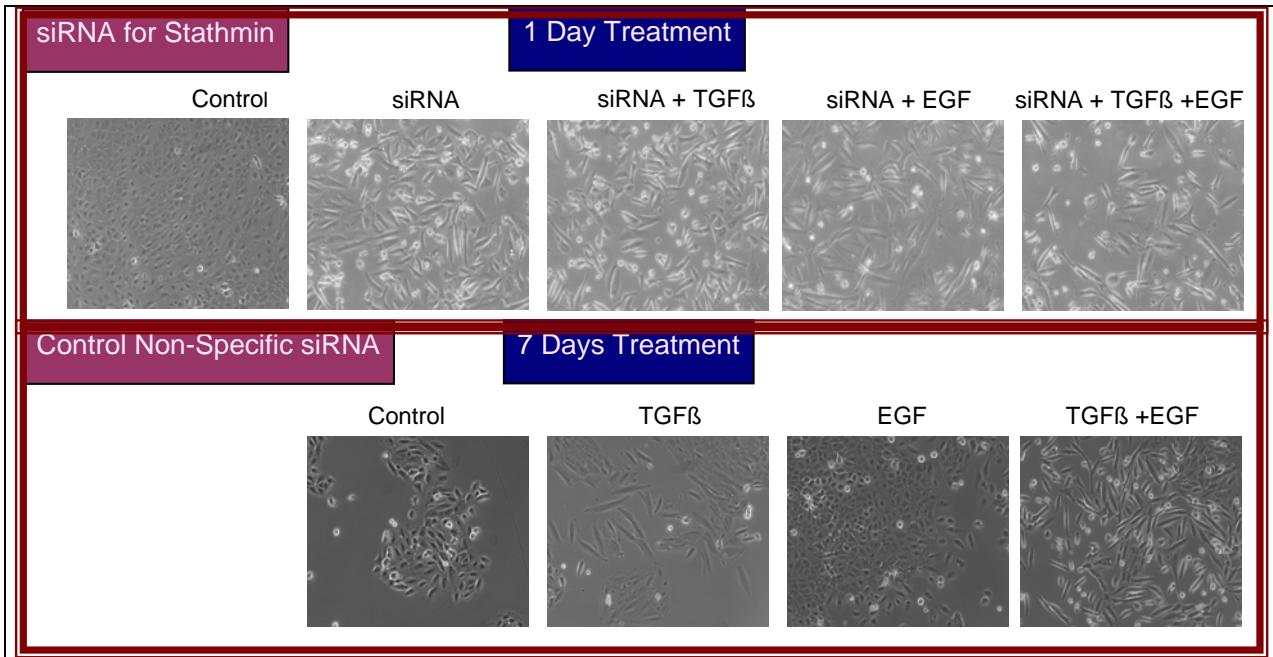


Figure 2. Stathmin expression is higher in adenocarcinoma regions of NeoTag2 tissue recombinants (B) compared to PIN lesions of NeoTag1 tissue recombinants (A).

adjacent normal glands (Fig. 2). In NeoTag2 tissue recombinants, stathmin expression was highest in the adenocarcinoma regions (Fig. 2).

The second main task was to investigate the effects of stathmin expression levels on the *in vitro*

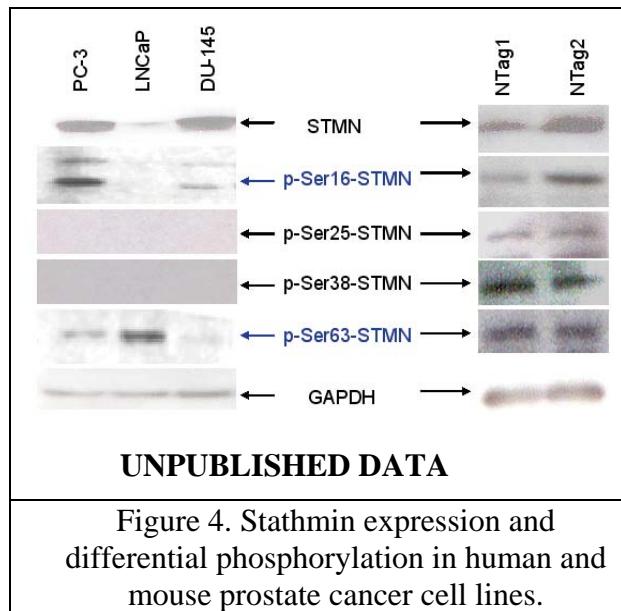
tumorigenic potential of NeoTag1 and NeoTag2 cells. In a recent work, Mistry et al. showed that stathmin expression levels modulated LNCaP tumorigenicity by suppressing clonogenic potential of the cells, increasing apoptosis and causing cell cycle arrest cells [1]. We looked at other effects of stathmin down-regulation in PCa cells, successfully knocked down stathmin in PC-3, DU145 and LNCaP cells in addition to NeoTag1 and NeoTag2 cells. Since stathmin is a microtubule binding protein, one of the first things we examined were changes in cellular morphology. Surprisingly, we found that knocking down stathmin can elicit an EMT-like phenotype in the DU145 cells. Of all the cell lines tested, DU145 is the only cell line that undergoes TGF β -induced epithelial to mesenchymal transition. Knocking down stathmin brought about a spontaneous epithelial to mesenchymal transition of the DU145 cells which mimics the transition seen in these cells in response to TGF β treatment. When DU145 cells are treated with TGF β , the cells attain an EMT like phenotype after 7 days. However, when stathmin was knocked down by siRNA, cells underwent epithelial to mesenchymal transition within twenty-four hours (Fig. 3). The epithelial to mesenchymal transition is associated with a change in cell shape and loss of cellular adhesion. These two phenotypes, taken together, suggest increased cell motility. Indeed epithelial to mesenchymal transition is essential for wound healing, cell migration and has been implicated in cell invasion during cancer progression and metastasis. Our preliminary results indicate that stathmin may modulate this epithelial to mesenchymal transition in DU145 cells. Presently, we are working on dissecting out the pathways by which stathmin carries out this function.



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Figure 3. Knocking down of stathmin in DU145 cells induces spontaneous epithelial to mesenchymal transition.

In Specific Aim II, the goal was to determine which signaling pathways are activated through selective phosphorylation of stathmin and whether inactivation of these pathways promotes sensitization to treatment with Taxotere or Erbitux. We found that in NeoTag1 and NeoTag2 cell lines, all four N-terminal serine residues (16, 25, 38 and 63) are phosphorylated (Fig 4). Cell lysates from these two cell lines were run on a 10% SDS gel and specific phospho-antibodies designed against serine-phosphorylated stathmin was used to determine stathmin phosphorylation in a Western Blot analyses. As proposed in the grant, we tested specific kinase inhibitors to determine if we could block phosphorylation at these residues.



Unfortunately, none of these small molecule inhibitors succeeded in knocking down stathmin phosphorylation. Hence, as an alternative approach, we designed primers to make phosphorylation site mutants of stathmin. We are now in the process of making a series of phosphorylation site mutant constructs which will give us either hypo- or hyper-phosphorylated stathmin upon transfection in

the cells. This will enable us to carry out the experiments proposed in the grant. For hyper-phosphorylated form of stathmin we are making serine to glutamine mutations at the four serine residues either alone or in different combinations (Fig 5). Similarly, for hypo-phosphorylated stathmin, we are making serine to alanine mutations at the four serine residues either alone or in different combination (Fig 5). Since we knocked down stathmin in the PC-3, DU145 and LNCaP cells in addition to NeoTag1 and NeoTag2 cells, we decided to study phosphorylation patterns in these cells lines also in addition to NeoTag1 and NeoTag2 cells. Interestingly, phosphorylation pattern in human cells differed from the mouse cells (Fig 4). In human PC-3, DU145 and LNCaP cells, stathmin phosphorylation was found only on serines 16 and 63 (Ser16 and Ser63) (Fig 4). Stathmin was not phosphorylated on Ser25 and 38. Furthermore, Stathmin was phosphorylated at Ser16 at

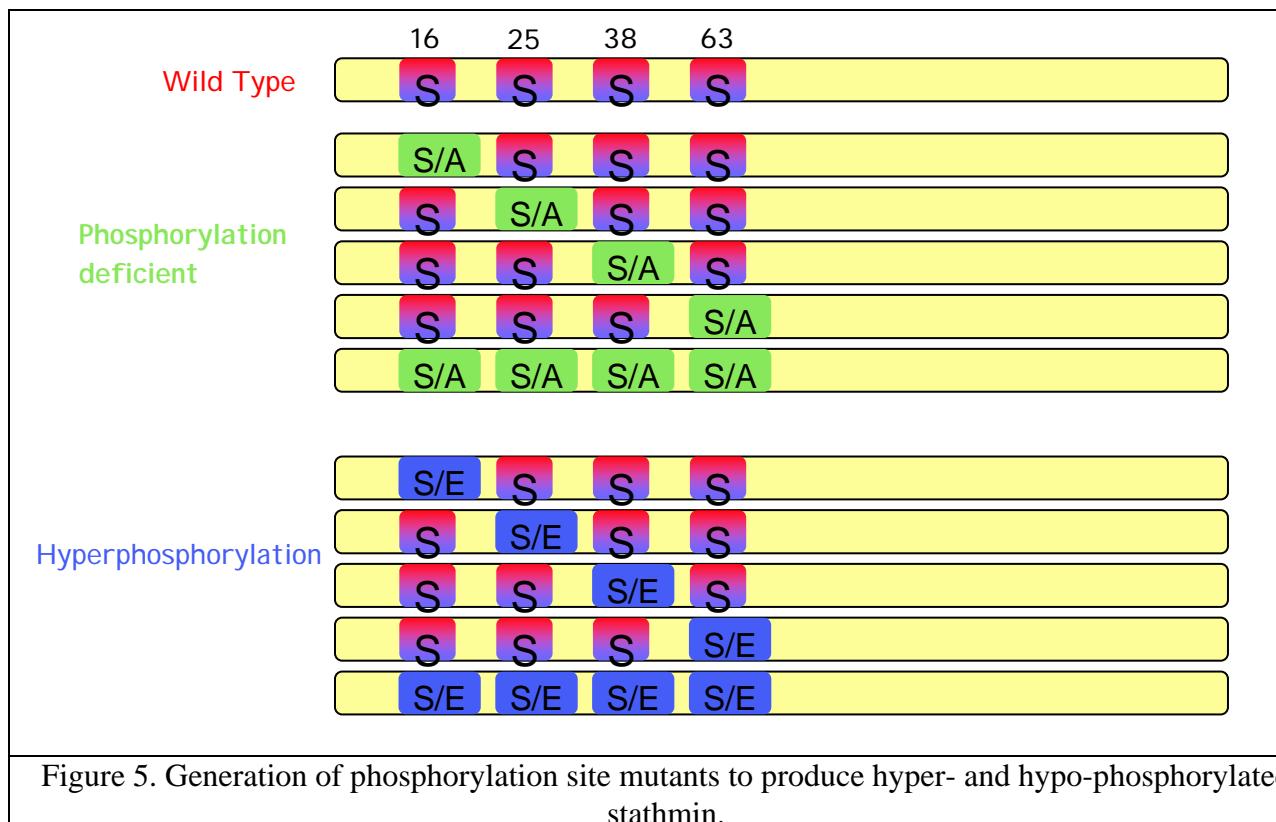


Figure 5. Generation of phosphorylation site mutants to produce hyper- and hypo-phosphorylated stathmin.

higher levels in androgen-independent PC-3 and DU145 cells, with higher levels in PC-3 cells compared to DU145 cells (Fig 4). Little to no phosphorylated Ser16 was detected in androgen-dependent LNCaP cells. Conversely, Ser63 phosphorylation of stathmin was highest in LNCaP cells compared to PC-3 and DU145 cells (Fig 4). This is an important observation as we can now start dissecting out pathways activated by stathmin in androgen-dependent vs androgen-independent prostate cancer cells. Differential phosphorylation of Ser16 in PC-3 and DU-145 cells and Ser63 in LNCaP cells suggests that stathmin may possess different roles in androgen-independent compared to androgen-dependent PCa. Androgens regulate normal prostate development and they promote the development of PCa. Even during androgen deprivation therapy when androgen levels are biochemically reduced, the androgen receptor (AR) signaling pathway may still be activated through other mechanisms. To elucidate which stathmin serine residues were phosphorylated in response to androgen and anti-androgen treatment, AR-positive

LNCaP cells were treated with vehicle (EtOH), DHT (10^{-8} M), Hydroxyflutamide (10^{-5} M), Casodex (10^{-5} M) and a combination of DHT and hydroxyflutamide or DHT and Casodex for twenty-four hours. Protein was extracted and stathmin phosphorylation at Ser16 and Ser63 was analyzed by Western blot analysis. Ser16 phosphorylation increased 2-fold with DHT treatment

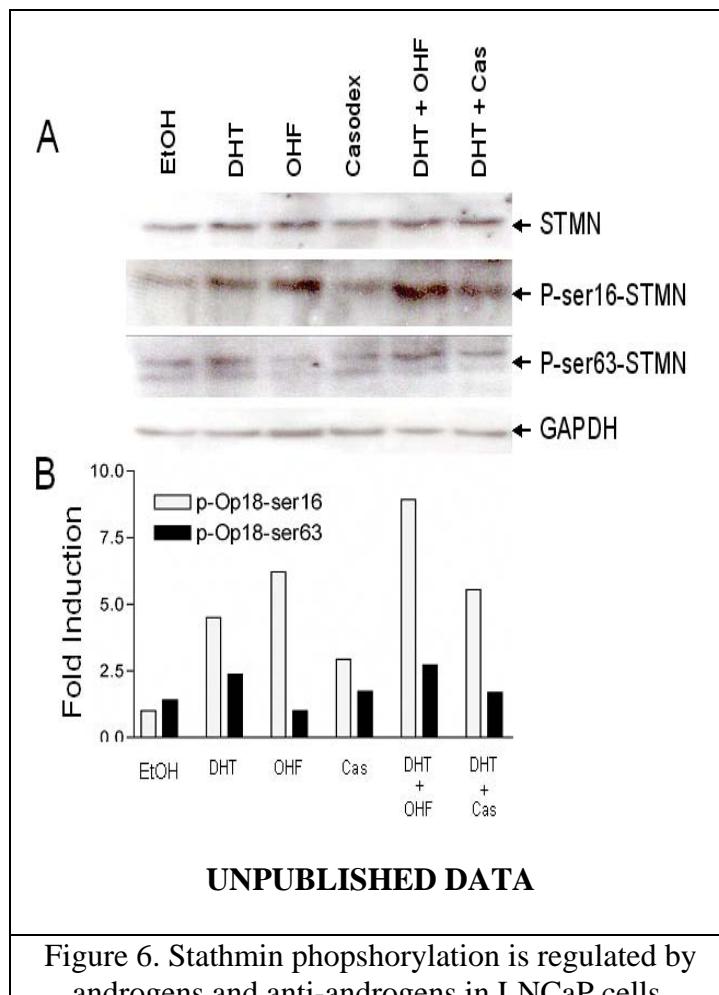


Figure 6. Stathmin phosphorylation is regulated by androgens and anti-androgens in LNCaP cells.

and 3.4-fold with hydroxyflutamide treatment (Fig. 10). In presence of both DHT and hydroxyflutamide, Ser16 phosphorylation increased \sim 9-fold indicating that DHT and hydroxyflutamide in combination resulted in a synergistic effect (Fig. 10). However, treatment with Casodex increased Ser16 phosphorylation marginally over the EtOH control and did not cause a synergistic or additive effect in combination with DHT treatment. Interestingly, changes in Ser63 phosphorylation were not as

pronounced as Ser16 phosphorylation in response to androgen and anti-androgen treatment. Stathmin phosphorylation at Ser63 only increased \sim 1.6 fold in response to DHT treatment and \sim 1.8 fold in response to combination treatment of DHT and hydroxyflutamide treatment. The remaining treatment groups caused little or no induction in Ser63 phosphorylation (Fig. 10). Although the biological significance of this increased phosphorylation in LNCaP cells is not

known at this time, inhibition of stathmin phosphorylation has been reported to promote G₂/M arrest in leukemic K562 cells. Future studies will determine which pathways are activated in PCa by this enhanced phosphorylation of stathmin by androgens and anti-androgens. Future studies will also elucidate whether or not these enhanced phosphorylations confer a survival advantage on cells by promoting progression of cell cycle and hence provide the cells with one of many mechanisms to become androgen independent.

Research Goals/ Training Accomplishments

1. In-depth immunohistochemical and statistical analyses on a tissue array containing human BPH and prostate cancer patients.
2. RNA interference technology to knock down stathmin in a variety of human and mouse prostate cancer cell lines.
3. Designing primers to introduce site-directed mutagenesis to generate hyper- and hypo-phosphorylated stathmin.

Reportable Outcomes

1. Manuscript in Preparation
 - a. **Increased Expression and Differential Phosphorylation of Stathmin may Promote Prostate Cancer Progression**
2. Abstract Presentation
 - a. **Stathmin in Prostate Cancer Development and progression, SBUR Fall Annual Meeting in Miami, December 2005**

Conclusions

The work is proceeding according to schedule. The limitation has been the failure of the kinase inhibitors in blocking stathmin phosphorylation on specific residues. However, designing primers to make hypo- and hyper-phosphorylated stathmin will allow us to perform the experiments outlined in the proposal. Another limitation has been the quality of staining in the immunohistochemical analyses involving the tissue array containing specimens from 111 prostate cancer patients. We acquired new set of tissue array from Vancouver General Hospital, Canada. Immunohistochemical analyses of stathmin expression yielded statistically relevant correlation between increasing Gleason Pattern and increasing stathmin expression. Work involving the tissue recombination model and the transgenic mice model have not yet been started and will be completed during year 2 and year 3.

Reference:

1. Mistry S.J., Bank A., and Atweh G. F. Targeting stathmin in prostate cancer. *Molecular Cancer Therapeutics*. 2005; 4(12): 1821-1829.